

Form PTO 1390 (REV 5-93)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER P50868
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/857580
INTERNATIONAL APPLICATION NO. PCT/US99/28992	INTERNATIONAL FILING DATE 07 December 1998	PRIORITY DATE CLAIMED 07 December 1999	
TITLE OF INVENTION A METHOD FOR QUANTITATIVE DETERMINATION OF AMINO ACIDS			
APPLICANT(S) FOR DO/EO/US Kalyan R. ANUMULA			

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98; and Form PTO-1449.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☒ Please amend the specification by inserting before the first line the sentence: This is a 371 of International Application PCT/US/99/28992, filed 07 December 1999, which claims benefit from the following Provisional Application 60/111,250, filed 07 December 1998.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ An Abstract on a separate sheet of paper.
19. ☐ Other items or information:

US APPLICATION NO. (if known) 09/857580 (37 CFR 1.50)		INTERNATIONAL APPLICATION NO. PCT/US99/28992		ATTORNEYS DOCKET NO. P50868	
20. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
Basic National Fee (37 C.F.R. 1.492(a)(1)-(5)):				690.00	
Search Report has been prepared by the EPO or JPO \$860.00					
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) \$690.00					
No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$710.00					
Neither International Preliminary Examination Fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$1,000.00					
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$690.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$0.00	
Claims	Number Filed	Number Extra	Rate		
Total claims	3 - 20 =	0	0 x \$18.00	\$0.00	
Independent claims	3 - 3 =	0	0 x \$80.00	\$0.00	
Multiple dependent claims (if applicable)			+ \$270.00	\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$690.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$	
SUBTOTAL =				\$690.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				\$	
TOTAL NATIONAL FEE =				\$690.00	
				Amount to be refunded	\$
				charged	\$

- a. ☐ A check in the amount of \$_____ to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 19-2570 in the amount of **\$690.00** to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-2570. A duplicate copy of this sheet is enclosed.
- d. ☒ General Authorization to charge any and all fees under 37 CFR 1.16 or 1.17, including petitions for extension of time relating to this application (37 CFR 1.136 (a)(3)).

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

GLAXOSMITHKLINE

Corporate Intellectual Property - UW2220

P.O. Box 1539

King of Prussia, PA 19406-0939

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SIGNATURE

Soma G. Simon

NAME

37,444

REGISTRATION NO.

PTU/PCT Rec'd 31 AUG 2001

Attorney Docket No. P50868

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant for DO/US : Kalyan R. Anumula
International App. No.: PCT/US99/28992
International File Date: 07 December 1999
Priority Date Claimed : 07 December 1998

Title of Invention : A Method for Quantitative Determination of Amino
Acids

BOX PCT

Assistant Commissioner for Patents
Washington, D.C. 20231
Attention: DO/US

SUPPLEMENTAL PRELIMINARY AMENDMENT

Sir:

Applicant respectfully requests that the following amendment and remarks be made of record prior to examination of the above-cited application.

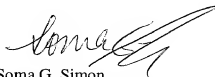
In response to the "Notification of Missing Requirements ... " mailed on 05 July 2001, and prior to the first Office Action on the merits, the Applicant requests entry of the following amendment. A copy of the "Notification of Missing Requirements ... " is enclosed.

Please consider, and add the enclosed Sequence Listing to the application file for the §371 application of PCT/US99/28992, which was mailed to the United States Receiving Office on June 7, 2001.

REMARKS

If any matter remains to be resolved before search, examination and allowance, or discussion of any matter will facilitate the prosecution of this application, the Examiner is invited to call the undersigned at the number provided.

Respectfully submitted,



Soma G. Simon
Attorney for Applicants
Registration No. 37,444

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N:\SOMA\CASES\p50868\suppprelim.doc

"EXPRESS MAIL CERTIFICATE"
"EXPRESS MAIL" MAILING LABEL NUMBER EL737849752US
DATE OF DEPOSIT June 7, 2001

Attorney Docket No. P50868

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant for DO/US : Anumula, et al.
International App. No.: PCT/US99/28992
International File Date: 07 December 1999
Priority Date Claimed : 07 December 1998

Title of Invention : A Method for Quantitative Determination of Amino
Acids

Docket No. : P50868

Honorable Commissioner of Patents
Box PCT
Washington, D.C. 20231

FIRST PRELIMINARY AMENDMENT

Sir:

Applicants respectfully request that the following amendments and remarks be made of record prior to examination of the above-cited application.

IN THE SPECIFICATION:

Please insert the following Abstract of the Disclosure on a separate page:

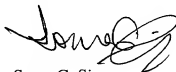
ABSTRACT OF THE DISCLOSURE

A novel method for the determination of amino acids by HPLC using pre-column derivatization is described. In this procedure, the amino acids are derivatized with 2-chlorobenzoxazole to yield highly fluorescent N-(2-benzoxazolyl)-amino acids (BOX-AAs) for detection at very high sensitivity. Derivatives can also be detected using conventional UV detection methods. The BOX-AAs can be separated on a C18 reversed phase column for quantitative estimation. This method can be used for the preparation of N-(2-benzoxazolyl)-amino acids in large amounts.

REMARKS

If any matter remains to be resolved before search, examination and allowance, or discussion of any matter will facilitate the prosecution of this application, the Examiner is invited to call the undersigned at the number provided.

Respectfully submitted,



Soma G. Simon
Attorney for Applicants
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n:\somatcases\50864\prelim.doc

A Method for Quantitative Determination of Amino Acids

Field of the Invention:

- 5 The present invention is directed to methods for the quantitative determination of amino acids.

Summary of the Invention:

- 10 The present invention is directed to a novel method for derivatizing amino acids or peptides, which method comprises reacting an amino acid or peptide with a fluorescent benzoxazole derivative, such as 2-chlorobenzoxazole.

- The present invention is also directed to a method of detecting, qualitatively and quantitatively, amino acids and peptides, which method comprises reacting an amino acid or peptide with 2-chlorobenzoxazole and detecting the resultant derivative by a suitable method, such as UV or fluorescence methods.
- 15

- The present invention also relates to the novel benzoxazole- amino acid derivatives, N-(2-benzoxazolyl) amino acids as described herein.
- 20

Brief Description of the Drawings:

- Figure 1 provides for the results of amino acid separation of N-(2-benzoxazolyl)-amino acids on a Beckman C18 column. The amino acids in the chromatogram were identified by derivatizing and analyzing the commercial amino acids individually.
- 25

Detailed Description of the Invention:

- Currently, amino acid analysis is routinely performed using HPLC with pre- and post-column derivatization chemistry for enhanced sensitivity. The present invention is directed towards the use of highly fluorescent N-(2-benzoxazole) derivatives, in particular 2-chlorobenzoxazole (CBOX) as a sensitive, fluorescent tag for the quantitative determination of amino acids. CBOX is readily available from commercial sources, such as Aldrich Chemicals.
- 30

- The present invention is directed towards use of the fluorescent N-(2-benzoxazolyl)-amino acids (BOX-AAs) derivatives in determination of the amino acids by HPLC using pre-column derivatization as described herein. In this procedure, the amino acids are derivatized with the CBOX to yield the highly fluorescent N-(2-benzoxazolyl)-amino acids (BOX-AAs) for detection at very high sensitivity. The derivatives can also be detected using conventional UV detection methods. The BOX-AAs can be separated on a C18 reversed phase columns for quantitative estimation. This method can be used for the preparation of N-(2-benzoxazolyl)-amino acids in large amounts.
- 35
- 40

Generically the amino acid standards and samples are derivatized with a BOX reagent, preferably CBOX in an alkaline medium, such as sodium carbonate, to yield a stable fluorescent amino acid derivative, which is separated by reversed phase chromatograph (Ultrasphere-ODS, 0.4 x 25 cm, Beckman). All of the BOX-AA are baseline resolved within 35 minutes or so. Peak areas for the BOX-AA are similar except for Pro (45%), Tyr and His (60%) and Lys (200%), presumably due to an additional amine group.

The invention will now be described by reference to the following examples which are merely illustrative and are not to be construed as a limitation of the scope of the present invention. All temperatures are given in degrees centigrade and all solvents are highest available purity.

Experimental

Preparation of the derivatizing solution

An aqueous solution of 0.1-5% sodium acetate tri-hydrate (w/v) was prepared first. Fifty μ L of this solution is mixed with 1.0 mL of methanol. 2-Chlorobezoxazole (10-50 μ L) was mixed with methanol-sodium acetate solution.

Derivatization of amino acids

Amino acid standards (1.0 nmol each from Pierce) in 50 μ L of buffer (for e.g. 0.25 M sodium carbonate) was mixed with 100 μ L of the above 2-chlorobezoxazole solution for derivatization. The reaction was allowed to continue typically at 80 $^{\circ}$ C for 10-60 min. After the reaction, the samples were diluted with sodium acetate solution and an aliquot was injected onto HPLC for analysis.

Proteins were hydrolyzed with 6N hydrochloric acid and the dried hydrolysates were derivatized in a manner similar to amino acid standards.

Separation of amino acids

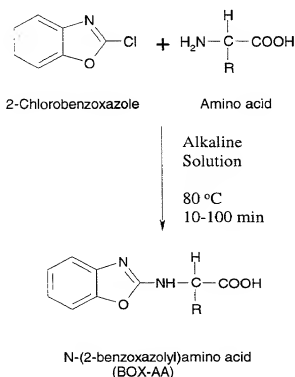
The derivatized amino acids were separated on a C18 reversed phase column. Typical solvent consisted of A: 2% ammonium bicarbonate (0.1-2% w/v) in 20% methanol-water and B: 1-20% methanol in acetonitrile. The amino acids were separated with a gradient generated from these solvents. A typical gradient consisted of 5%B isocratic for 8 min followed by a linear gradient to 45%B over 35 min.

Results

Reaction scheme for amino acid derivatization with 2-chlorobenzoxazole to yield 2-benzoxazole derivatives is shown in Scheme I.

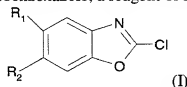
2-Benzoxazole derivatives can be detected using either UV or fluorescence. An excitation and emission maxima of 245 nm and 320 nm respectively were used for

- 5 fluorescence detection. Separation of BOX-AAs on a Beckman C18 (ODS Ultrasphere, 0.46 x 25 cm) is shown in Figure 1.



Fluorescence: 245 nm ex. and 320 nm em.

- 10 As an alternative to 2-Chlorobenzoxazole, a reagent of formula (I) may also be used:



wherein R₁ and R₂ are independently selected from the group consisting of hydrogen, halo and C₁₋₄hydroxy.

Scheme I. Reaction scheme for the formation of 2-benzoxazolyl-AAs.

15

Another aspect of the present invention are the specific derivatized amino acid derivatives with a benzoxazole derivative, i.e., N-(2-benzoxazolyl) amino acids. The commonly used, well known amino acids encompassed by this description include:

- 20 Abbreviation Amino acid name

	Ala	Alanine
	Arg	Arginine
	Asn	Asparagine
	Asp	Aspartic Acid
5	Asx	Aspartic Acid or Asparagine
	Cys	Cysteine
	Glu	Glutamic Acid
	Gln	Glutamine
	Glx	Glutamine or Glutamic Acid
10	Gly	Glycine
	His	Histidine
	Ile	Isoleucine
	Leu	Leucine
	Lys	Lysine
15	Met	Methionine
	Phe	Phenylalanine
	Pro	Proline
	Ser	Serine
	Thr	Threonine
20	Trp	Tryptophan
	Tyr	Tyrosine
	Val	Valine

- 25 All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.
- 30 The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the area can, using the preceding description, utilize the present invention to its fullest extent. Therefore, the Examples herein are to be
- 35 construed as merely illustrative and not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.

What is Claimed Is:

1. A method of derivatizing amino acids or peptides, which method comprises
5 reacting an amino acid or peptide with 2-chlorobenzoxazole.
2. A method of detecting amino acids and peptides, which method comprises
reacting an amino acid or peptide with 2-chlorobenzoxazole and detecting the
resultant derivative by UV or fluorescence methods.
- 10 3. N-(2-benzoxazolyl) amino acids.

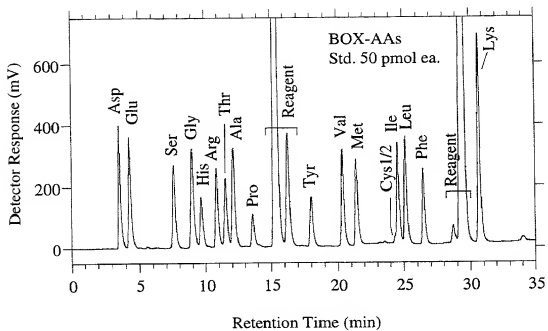


FIGURE 1

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

"A Method for Quantitative Determination of Amino Acids"

the specification of which (check one)

☐ is attached hereto.

☒ was filed on **7 December 1999** as Serial No. **PCT/US99/28992**
and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or Inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed
--------	---------	-------------	------------------

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date
60/111,250	7 December 1998

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Serial No.	Filing Date	Status
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I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

Customer Number 20462.

Address all correspondence and telephone calls to Soma G Simon, SmithKline Beecham Corporation, Corporate Intellectual Property-U.S., UW2220, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939, whose telephone number is 610-270-5019.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Inventor: 1-10 Kalyan R ANJUMULA

Inventor's Signature: Kalyan R. Anjumula Date: 06 Jan 2000

Residence: 624 Jamie Circle, King of Prussia, Pennsylvania 19406 PA

Citizenship: United States of America

Post Office Address: SmithKline Beecham Corporation
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SEQUENCE LISTING

<110> ANUMULA, KALYAN R.

<120> A METHOD FOR QUANTITATIVE DETERMINATION
OF AMINO ACIDS

<130> P50868

<140> 09/857,580

<141> 2001-06-07

<150> PCT/US99/28992

<151> 1999-12-07

<150> 60/111,250

<151> 1998-12-07

<160> 2

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Ser Thr Trp Tyr Val

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<210> 2

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> Amino acids derived from
2-chlorobenzoxazole to yield highly fluorescent
N-(2-benzoxazolyl)-amino acids for detection at
very high sensitivity.

<400> 2

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1

5

10

15

Lys